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Photoprotective, antibacterial activity and determination of phenolic compounds of *Neoglaziovia variegata* (Bromeliaceae) by high performance liquid chromatography-diode array detector (HPLC-DAD) analysis

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The photoprotective activity of flowers and antibacterial activity of the leaves of *Neoglaziovia variegata* were investigated. This study also identified the first phenolic compounds of the extracts and fractions of this species by high performance liquid chromatography (HPLC). The photoprotective activity was measured using the spectrophotometric method. The antibacterial activity was evaluated by microdilution method. The chromatographic analysis was performed in HPLC coupled with iode array detector (DAD) detector, using wavelengths of 254 and 320 nm. Nv-Fl-EtOH, Nv-Fl-$CHCl_3$ and Nv-Fl-AcOEt showed characteristic absorption bands in regions UVB and UVA in a concentration-dependent manner. Nv-Fl-$CHCl_3$ presented the highest sun protection factor (11.45 ± 2.87). Extracts from the leaves showed activity against most of the microorganisms tested, especially *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica*, *Serratia marcescens* and *Shigella flexneri*. HPLC analysis revealed the presence of six phenolics: two flavonoids (isoquercetin and kaempferol-3-*O*-rhamnoside) and four phenolic acids (caffeic, protocatechuic, *p*-coumaric and vanillic acids). This result justifies the biological properties, that the species has demonstrated and can lead other phytochemical studies aiming the isolation of these compounds.

Key words: Bromeliaceae, *Neoglaziovia variegata,* photoprotective activity, antibacterial activity, phenolic compounds.

INTRODUCTION

Currently, the damage caused by ultraviolet radiation (UV) has become the focus of several studies. Oxidative

stress and inflammatory responses induced by UV radiation can cause a variety of harmful effects in skin, including premature photoaging and the induction of immunosupression and skin carcinogenesis (Vilela et al., 2011). For this reason, the use of natural active ingredients in photoprotective formulations has been increasingly reported in the literature as an alternative to the use of chemical filters. Some studies have focused on the use of plant extracts or fractions containing phenolic compounds, especially flavonoids, due to their antioxidant and photoprotective potential (Oliveira-Júnior et al., 2013a).

Recurring epidemics of drug resistant bacterial diseases such as those caused by mycobacteria (tuberculosis and non-tuberculous infections), staphylococci (methicillin-resistant *Staphylococcus aureus* or MRSA infections) and various Gram-negative enterobacteria (enterobacterial infections) have reinforced the need to search for alternative antimicrobials. In this context, the screening of plant extracts has been of interest to scientists for the discovery of new compounds effective in the treatment of bacterial infection (Yadav et al., 2012; Souza et al., 2012).

The Bromeliaceae family is predominantly neotropical, and comprises 58 genera and approximately 3172 species (Luther, 2008). The phytochemistry of this family is characterized by the presence of flavonoids, triterpenoids, steroids, diterpenes, cinnamic acid derivatives, lignans, nitrogen compounds among others (Manetti et al., 2009).

Some studies have demonstrated that Bromeliaceae species have several pharmacological properties, such as antinociceptive (Lima-Saraiva et al., 2012a), antiulcer (Carvalho et al., 2010), antioxidant (Santana et al., 2012), photoprotective (Oliveira-Junior et al., 2013a) and antibacterial (Santana et al., 2012; Oliveira-Junior et al., 2012) activities. These studies correlate the biological properties of these species with their total phenolic and total flavonoid content.

In Brazil, *Neoglaziovia variegata* is popularly known as “caroá”. This species can be found in the Brazilian Caatinga vegetation, in several states of the country, but especially in the Northeastern region (Pereira and Quirino, 2008). Previous study conducted by our research group demonstrated that the ethanolic extract from the leaves of *N. variegata* presents gastroprotective (Machado et al., 2013) and antinociceptive (Lima-Saraiva et al., 2012a) activities. It was also observed that extracts and fractions from the leaves of *N. variegata* have antioxidant (Lima-Saraiva et al., 2012b) and photoprotective (Oliveira-Junior et al., 2013b) properties. Furthermore, extracts and fractions obtained from flowers of *N. variegata* also showed antioxidant and antibacterial activities (Oliveira-Junior et al., 2012).

These pharmacological properties are probably related to the high content of phenols and flavonoids determined (Manetti et al., 2009; Teng et al., 2013; Diniz et al., 2013; Silva et al., 2013). However, no compound has been identified for this species to date. Therefore, the aim of this study was to continue investigating the biological and chemical potential of *N. variegata*. For this, the photoprotective activity from flowers and antibacterial activity from leaves of this plant were reported; besides the identification of phenolic compounds present in ethanol extracts and hexane, chloroform and ethyl acetate fractions were done from leaves and flowers of *N. variegata* using the technique of high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Plant

The leaves and flowers of *N. variegata* (Arruda) Mez were collected in the city of Petrolina (Coordinates: S 08°59'16"; W 40°35'20"), State of Pernambuco, Brazil, in January of 2012. The samples were identified by a botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (6441) was deposited at the Herbário do Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco (UNIVASF).

Extraction

The leaves (450 g) dried and powdered were macerated with 95% ethanol at room temperature (25°C) for 72 h. The extractive solution was concentrated under vacuum in a rotatory evaporator oven at 45°C, producing 273 g of crude ethanol extract (Nv-Le-EtOH). The Nv-Le-EtOH was suspended in a mixture of H_2O:MeOH (7:3) and extracted successively with hexane (Hex), chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in crescent order of polarity to obtain the respective fractions: Nv-Le-Hex, Nv-Le-$CHCl_3$ and Nv-Le-AcOEt. The same was performed with the flowers dried and powdered (1174 g), resulting in 143 g of crude ethanol extract (Nv-Fl-EtOH) and posteriorly in the fractions (Nv-Fl-Hex, Nv-Fl-$CHCl_3$ and Nv-Fl-AcOEt).

Photoprotective activity

The photoprotective activity of the extract and fractions of the flowers of the plant was measured by determination of the maximum absorption wavelength (λ_{max}) and sun protection factor (SPF) *in vitro*. For this, the samples were diluted in absolute ethanol, obtaining concentrations of 5, 25, 50 and 100 mg/L (in triplicate). Subsequently, spectrophotometric scanning was performed at wavelengths between 260 and 400 nm, with intervals

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Table 1. Normalized product function used in the calculation of SPF.

Wavelength (nm)	EE × I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	1.0000

EE: Erythemal effect spectrum; I: solar intensity spectrum.

of 5 nm. The readings were performed using 1 cm quartz cell and ethanol used as blank with a qualified equipement (Violante et al., 2009). Calculation of SPF was obtained according to the equation developed by Mansur et al. (1986):

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

where EE (λ) is the erythemal effect spectrum; I (λ) is the solar intensity spectrum; Abs (λ) is the absorbance of sunscreen product; and CF is the correction factor (= 10). The values of EE × I are constants. They were determined by Sayre et al. (1979), and are as shown in Table 1.

Antibacterial activity

The antibacterial activity of the extract and fractions of the leaves of the plant was measured by determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Thus, the reference bacterial strains used in this study were obtained from National Institute of Quality Control in Health (INCQS/FIOCRUZ - Brazil). The microorganisms used were: *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 12022) and *S. aureus* (ATCC 25923).

The antibacterial effect was evaluated by the method of microdilution (Santos et al., 2012) as recommended by The National Committee for Clinical Laboratory Standards (CLSI, 2003). Initially, a stock solution of 25 mg/ml of the extract and fractions was prepared using an aqueous solution of 2.0% DMSO (v/v). 200 µl of this dilution was transferred to the microplate containing 200 µl of Müller-Hinton broth. Then, serial dilutions were performed resulting in concentrations of 12500, 6250, 3120, 1560, 780, 390, 190 and 95 µg/ml. The inoculum containing 5×10^5 CFU/ml (0.5 in McFarland scale) was added to each well. It was reserved in wells of microplate for sterility control of the broth, bacterial growth and action of antimicrobial reference (Gentamicin). An initial concentration of 1.6 mg/ml was used for gentamicin, which was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 µg/ml. The microplates were incubated under conditions aerobically for 18 to 24 h at 37°C when 10 µl of 2,3,5-triphenyl-tetrazolium (CTT) 2% were added to each well to detect the color change of the CTT (colorless) to red, reflecting the bacterial metabolism active. The MIC was defined as the lowest concentration of the extracts that visibly inhibited the bacterial growth. To determine the MBC, aliquots of 10 µl were withdrawn from each well containing the extracts and transferred to Petri plates containing agar Müller-Hinton. The plates were incubated for 24 h at 37°C. The appearance of bacterial colony for a given concentration indicates that it was not able to kill 99.9% or more bacterial inoculum used. Assays were performed in triplicate. The density of the extracts was employed to convert µl/ml in mg/ml. The latter being used to express the MIC and MBC.

HPLC analysis of phenolic compounds

Polyphenol identification analyses were carried out using a liquid chromatograph (Prominence LC-20AT, Shimadzu®, Japan) with a diode array detector (DAD, SPD-M20A) coupled to an LCSolution ChemStation data-processing station. The column used was a C18 Betasil (250 × 4.6 mm; 5 µm; Thermo Fisher Scientific, Runcorn, UK), operated at 27°C.

The mobile phase consisted of solvent A (ultrapure water and acetic acid, 99:1) and solvent B (methanol). The gradient (v/v) began with 38% of solvent B, rising to 70% at 20 min, 85% at 25 min, and 100% at 30 min, and remaining at this concentration for another 5 min. The injection volume was 20 µl, and the flow rate was 1 ml/min. Chromatograms for the phenolic compounds were recorded at 254 and 320 nm. The phenolic compounds were identified by comparing each identified compound with commercial standards using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards. The chemical standards were analysed by the same RP-HPLC-DAD method as phenolic extracts. Authentic markers available at the market were used for chromatographic comparison of data. Isoquercetin, gallic acid, protocatechuic acid, kaempferol-3-*O*-rhamnoside, vanillic acid and *p*-coumaric acid were supplied by Sigma-Aldrich® Chemie (Steinheim, Germany) for HPLC analysis with purity ≥99%. The HPLC analysis was performed with all the extracts and fractions of *N. variegata* (leaves and flowers).

Statistical analysis

The data obtained were analyzed using the GraphPad Prism® version 5.0 and expressed as mean ± standard deviation (SD) were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Photoprotective activity

Figure 1 shows the spectrophotometric absorption profile of the extract and fractions of the flowers of *N. variegata*. By analyzing the data, it can be observed that Nv-Fl-EtOH, Nv-Fl-$CHCl_3$ and Nv-Fl-AcOEt showed characteristic absorption bands in regions UVB and UVA in a concentration dependent manner, suggesting a possible photoprotective potential. The maximum absorption wavelength (λ_{max}) for Nv-Fl-EtOH, Nv-Fl-$CHCl_3$ and Nv-Fl-AcOEt was 285 (UVB) and 330 (UVA), 325 (UVA) and 330 nm (UVA), respectively. Nv-Fl-Hex did not demonstrate satisfactory absorption. This result complements the data obtained in previous experiments with extracts and fractions obtained from the leaves of *N.*

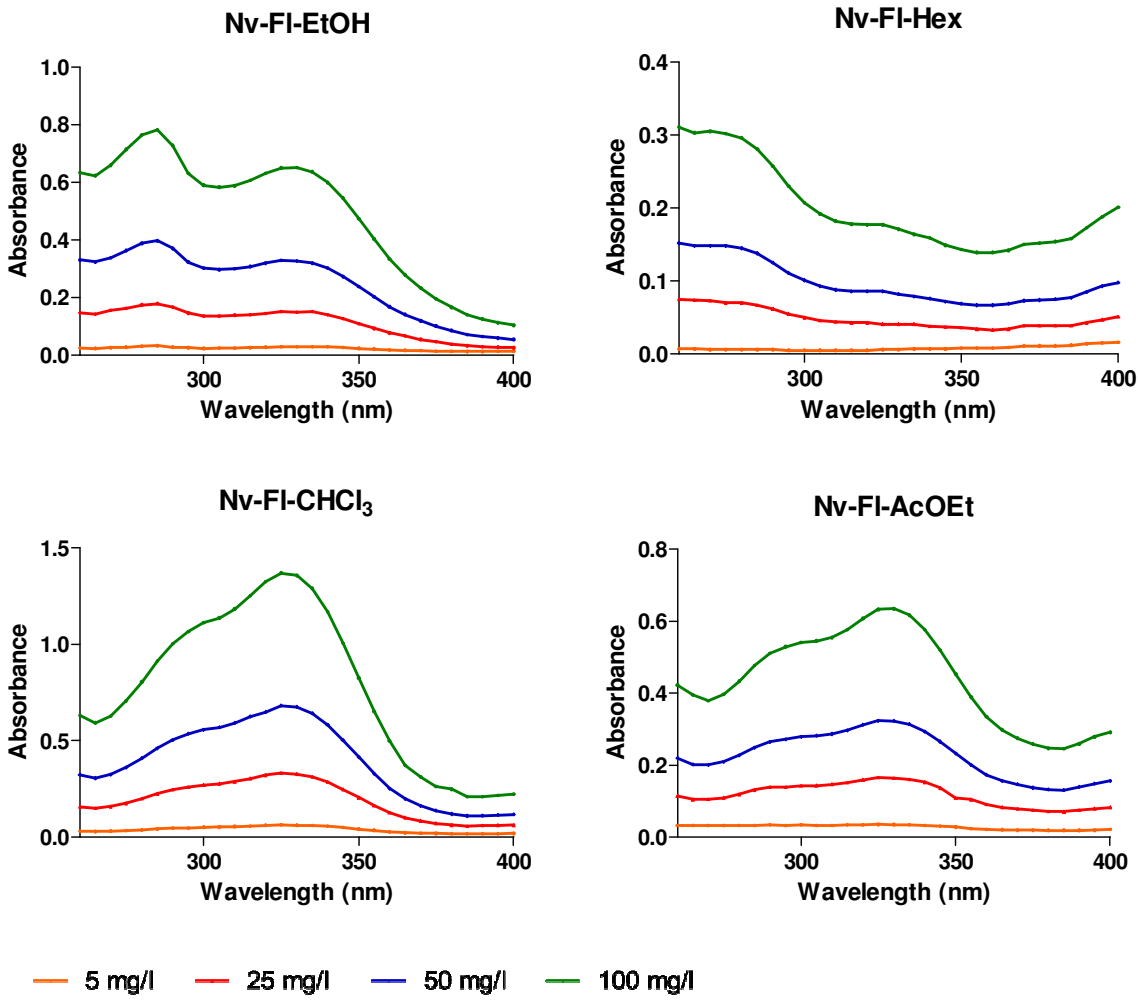

Figure 1. Spectrophotometric absorption profile of the extract and fractions of the flowers of *Neoglaziovia variegata* (260 to 400 nm).

variegata, confirming that this species has interesting photoprotective potential (Oliveira-Junior et al., 2013b).

Several studies have correlated the sunscreen activity of plants with its phenolic content, especially flavonoids (Silva et al., 2014; Souza et al., 2015). The protective effects of flavonoids in biological systems are justified by their ability to chelate metal catalysts, activate antioxidant enzymes, inhibit oxidases and their capacity to transfer electrons free radicals. Furthermore, flavonoids protect plants from solar UV radiation and scavenge UV generated ROS through differents photoprotection mechanisms, including UV absorption, direct and indirect antioxidant properties, and modulation of several signaling pathways (Saewan and Jimtaisong, 2013).

The SPF *in vitro* was determined by the spectrophotometric method developed by Mansur (1986) using the UVB region, considered to be the region of the greatest incidence during the day in which people are exposed to for long (Dutra et al., 2004). In Figure 2, it can be observed that Nv-Fl-$CHCl_3$ showed higher SPF at concentration 100 mg/L (11.45 ± 2.87), followed by Nv-Fl-EtOH and Nv-Fl-AcOEt (5.95 ± 1.65 and 5.48 ± 1.54, respectively). Nv-Fl-Hex showed low values of SPF at 100 mg/L (1.97 ± 0.33).

The results about SPF also showed standard concentration-dependent. Although the test has been carried out *in vitro*, it was demonstrated that this method correlates well with *in vivo* tests, because it relates the absorbance of the substance in question with the erythematogenic effect of radiation and intensity of light at specific wavelengths between 290 and 320 nm (UVB region) (Violante et al., 2009).

Antibacterial activity

Several studies have been conducted in order to obtain natural products from plants as an alternative to combat

Table 2. Determination of minimum inhibitory concentration (MIC) of the extract and fractions from the leaves of *Neoglaziovia variegata*.

Microorganisms	MIC (µg/ml)				
	Nv-Le-EtOH	Nv-Le-Hex	Nv-Le-$CHCl_3$	Nv-Le-AcOEt	GEN
Bacillus cereus	1560	*	*	780	0.4
Enterococcus faecalis	1560	3120	3120	3120	0.4
Escherichia coli	1560	3120	3120	780	*
Klebsiella pneumoniae	1560	3120	3120	3120	0.05
Salmonella enterica	780	3120	1560	1560	0.05
Serratia marcescens	390	1560	1560	1560	*
Shigella flexneri	390	3120	3120	780	*
Staphylococcus aureus	3120	3120	3120	780	0.025

*Absence of bacterial increase at all concentrations tested (n=3).

Table 3. Determination of minimum bactericidal concentration (MBC) of the extract and fractions from the leaves of *Neoglaziovia variegata*.

Microorganisms	MBC (µg/ml)				
	Nv-Le-EtOH	Nv-Le-Hex	Nv-Le-$CHCl_3$	Nv-Le-AcOEt	GEN
Bacillus cereus	1560	190	*	1560	0.4
Enterococcus faecalis	3120	3120	3120	3120	0.4
Escherichia coli	6250	3120	3120	3120	0.4
Klebsiella pneumoniae	3120	6250	6250	3120	0.05
Salmonella enterica	3120	3120	6250	6250	0.05
Serratia marcescens	1560	6250	6250	6250	0.025
Shigella flexneri	1560	3120	12500	12500	0.025
Staphylococcus aureus	3120	3120	3120	3120	0.025

*Absence of bacterial increase at all concentrations tested (n=3).

infections caused by antibiotic-resistant microorganisms (Cerqueira et al., 2011). Therefore, the antibacterial activity of the extract and fractions of *N. variegata* was evaluated against eight reference bacteria.

In relation to determination of the MIC (Table 2), the data showed that the strains were sensitive to all the extracts and fractions used, especially Nv-Le-EtOH and Nv-Le-AcOEt. These samples showed favorable bacteriostatic effect, especially against *B. cereus*, *E. coli*, *S. enterica*, *S. marcescens* and *S. flexneri*.

Determination of MBC showed results similar to the MIC (Table 3). All strains were sensitive to extract and fractions tested, mainly for Nv-Le-EtOH and Nv-Le-Hex. These samples showed favorable bactericidal effect, especially against *B. cereus* and *S. flexneri*. These results also complement the data obtained in previous experiments with extracts and fractions obtained from the flowers of *N. variegata*, confirming that this species also has significant antibacterial activity (Oliveira-Júnior et al., 2012).

Many classes of secondary metabolites are known for their antimicrobial activity. From these, the flavonoids are considered a promising source of new antibacterial agents. In most cases, these compounds are produced by plants as a defense mechanism against microbial infections. Thus, once extracted and isolated from plants, flavonoids are able to maintain their antimicrobial efficacy against a wide array of microorganisms. In addition, their antimicrobial activities against some Gram-negative and Gram-positive bacteria have been reported in many papers. Therefore, the flavonoids may be promising for new class compounds in antimicrobial therapy (Hendra et al., 2011; Özçelik et al., 2008).

HPLC analysis of phenolic compounds

Several techniques are in use to identify phenolic compounds such as thin layer chromatography (TLC), high performance thin layer chromatography (HP-TLC), gas chromatography, UV detection, high performance liquid chromatography (HPLC), and mass spectrometry (Khoddami et al., 2013). These techniques are useful to identify compounds whose structure is known from a

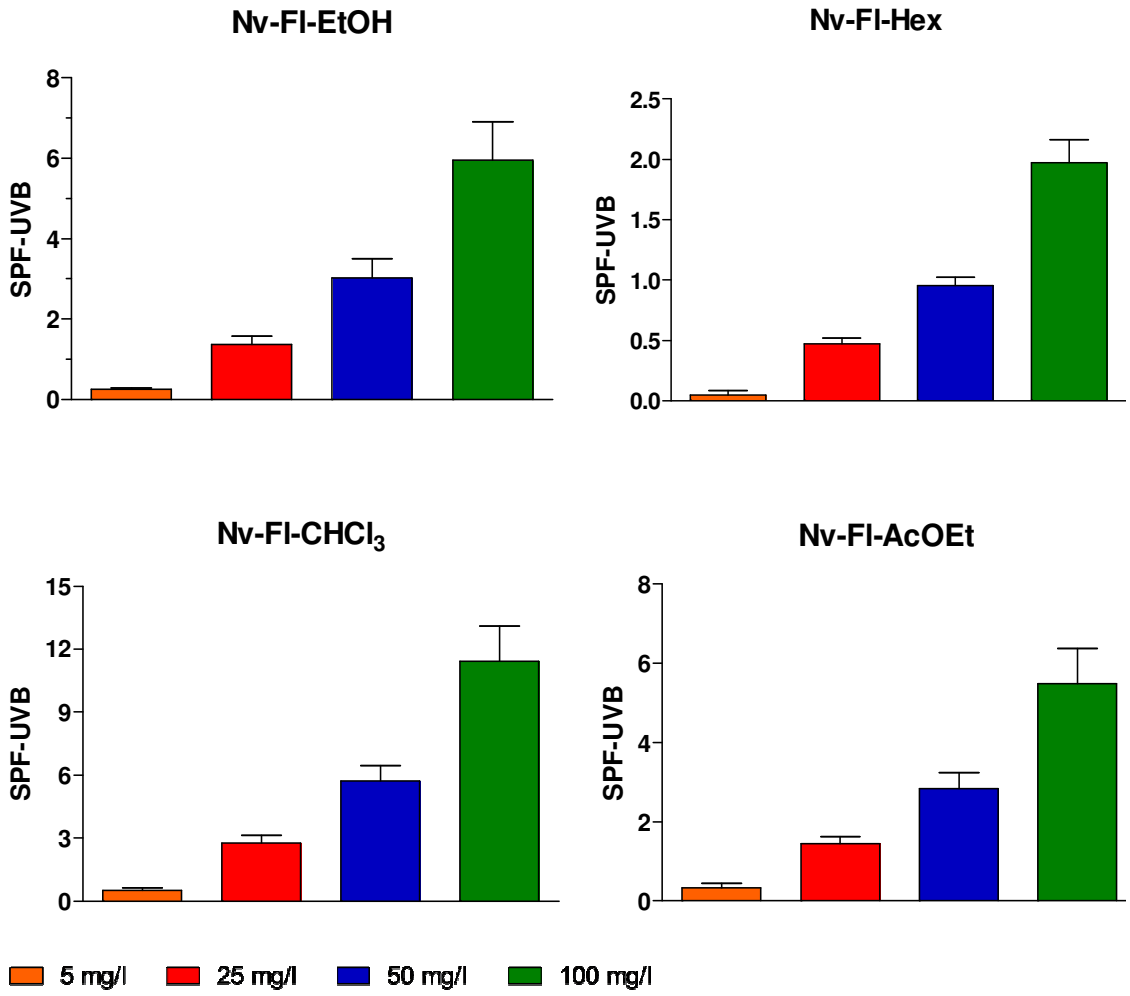

Figure 2. Sun Protection Factor (SPF-UVB) *in vitro* of the extract and fractions of the flowers of *Neoglaziovia variegata*.

mixture of substances (Lianda et al., 2012).

The composition profile of phenolic compounds of *N. variegata* was analyzed by HPLC-DAD. Based on the chromatograms expressed in Figure 3, it was possible to identify the presence of flavonoid isoquercetin in Nv-Le-EtOH, *p*-coumaric acid in Nv-Le-$CHCl_3$ and vanillic, *p*-coumaric and protocatechuic acids in Nv-Le-AcOEt. These compounds were identified by comparison with the parameters retention time and absorption bands in the ultraviolet spectra of the standards were used (Table 4).

Figure 4 shows the chromatograms obtained after analysis of the extract and fractions of the flowers of *N. variegata*. The chromatograms demonstrated the identification of several compounds such as isoquercetin (Nv-Fl-EtOH), vanillic acid (Nv-Fl-$CHCl_3$), isoquercetin, *p*-coumaric acid, vanillic acid, protocatechuic acid, caffeic acid and kaempferol-3-*O*-rhaminoside (Nv-Fl-AcOEt). However, the content of individual compounds was different.

It is known that flavonoid isoquercetin is present in several Bromeliaceae species such as *Portea petropolitana*, *Pitcairnia rubriflora*, *Pitcarnia sprucei* and *Vriesia regina* (Manetti et al., 2009; Williams, 1978). Thus, the identification of this compound in *N. variegata* can classify it as an important chemotaxonomic marker for the family Bromaliaceae.

In fact, flavonoids represent the main class of secondary metabolites from species of Bromeliaceae (Manetti et al., 2009; Queiroga et al., 2004). Besides the isoquercetin, this study also reports the identification of kaemferol-3-*O*-rhaminoside. These flavonoids have important pharmacological properties, which may be cited as atioxidant, antimicrobial, anti-inflammatory activities and hormones regulatory activity (Agrawal, 2011).

In addition to the flavonoids, same phenolic acids were also identified (Table 4). Some studies have shown that the presence of these phenolic acids is common in Bromaliaceae species, especially *p*-coumaric and caffeic

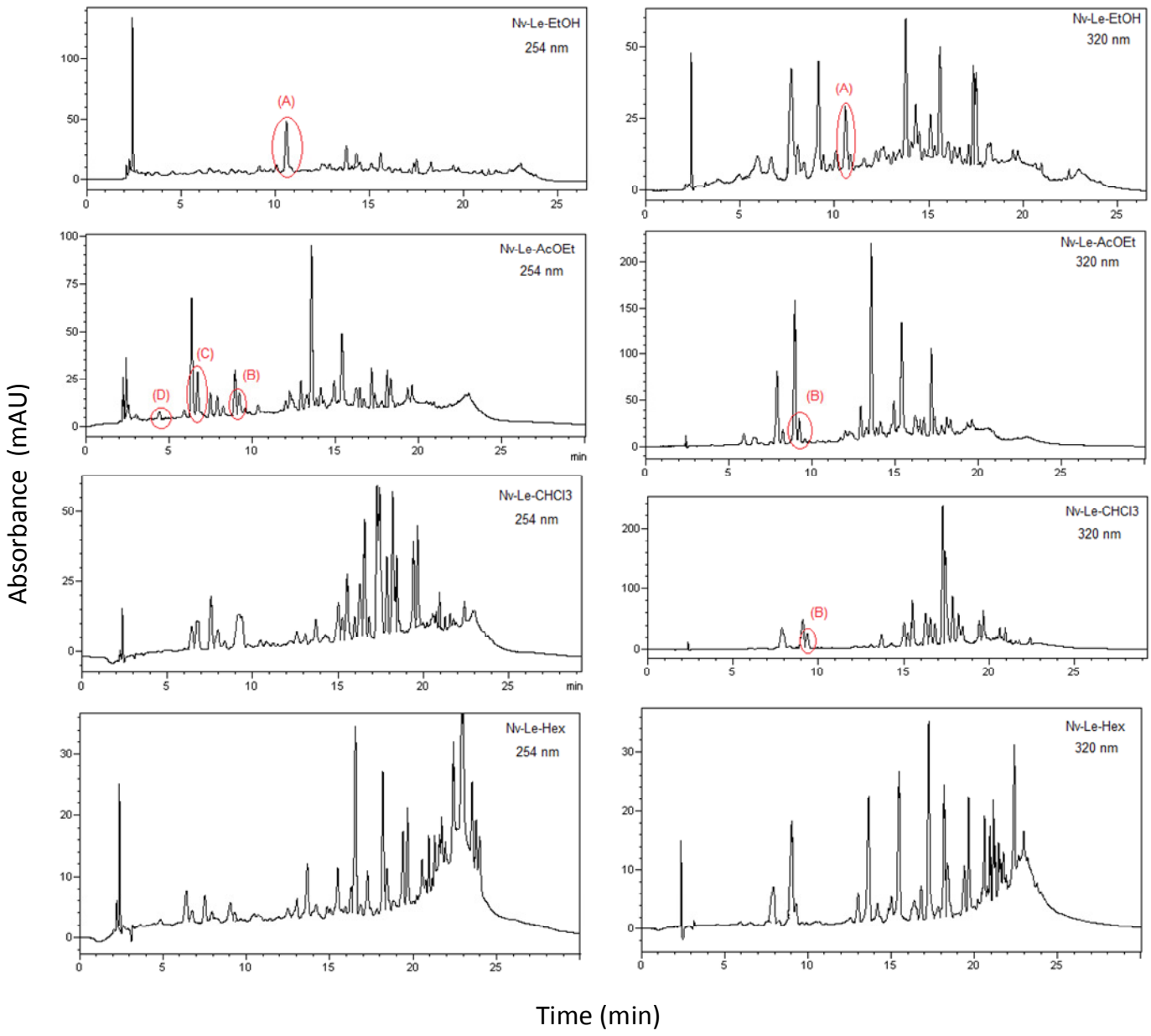

Figure 3. HPLC chromatograms of the extract and fractions from the leaves of *Neoglaziovia variegata*. Table 4 shows the peak identification.

Table 4. Retention times (t_R/min) and maximum absorption (λ_{max}/nm) of the phenolic standards and their correlation with the compounds of *Neoglaziovia variegata* (Peaks).

Peak	Phenolic compound	t_R/min	λ_{max}/nm
A	Isoquercetin	10.598	256, 355
B	*p*-Coumaric acid	8.813	310
C	Vanillic acid	6.736	262, 292
D	Protocatechuic acid	4.438	260, 295
E	Caffeic acid	6.499	324
F	Kaempferol-3-*O*-rhamnoside	12.353	265, 347

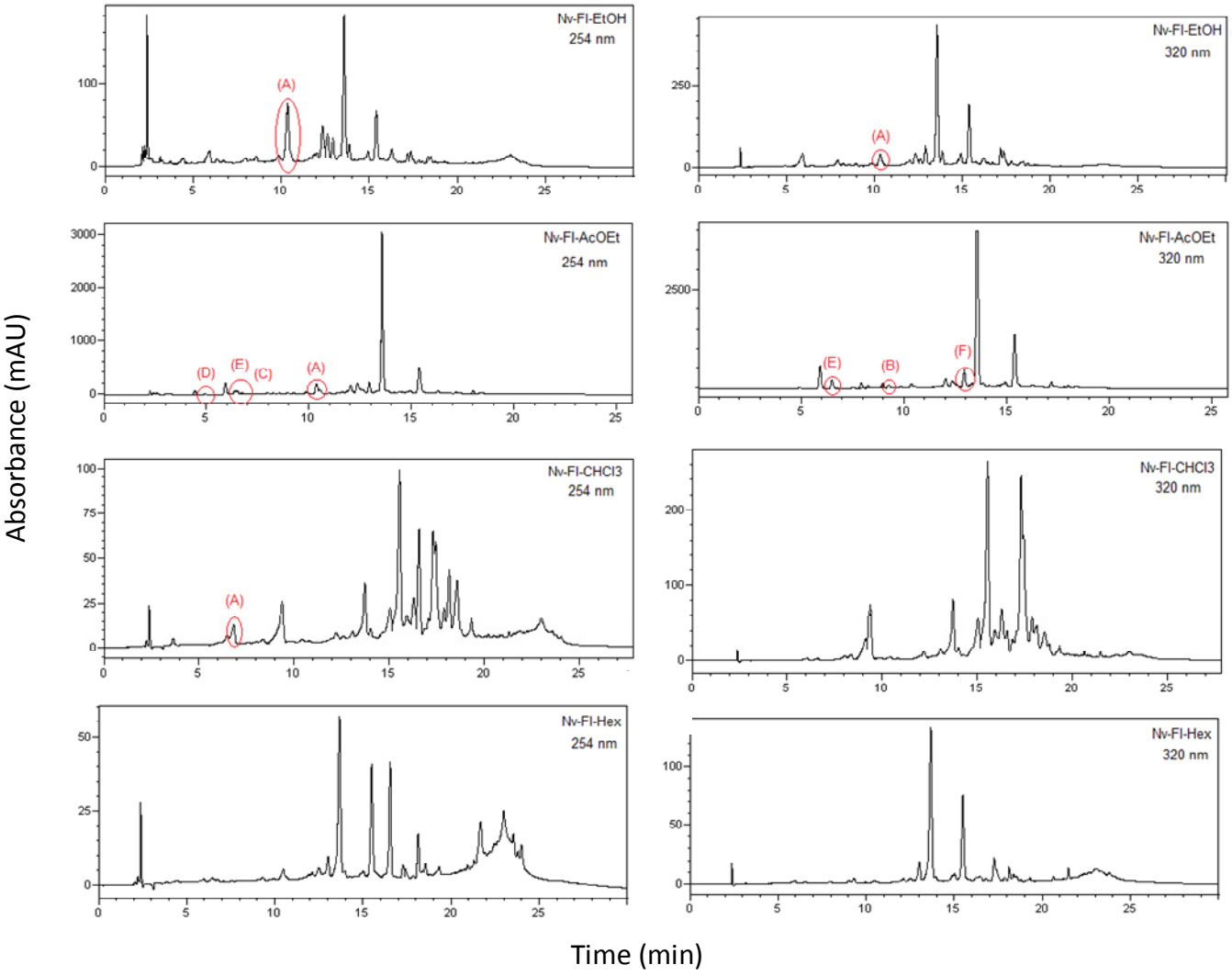

Figure 4. HPLC chromatograms of the extract and fractions from the flowers of *Neoglaziovia variegata*. Table 4 shows the peak identification.

acid (Sutherland and Gortner, 1958; Mullen et al., 2007; Raffauf et al., 1981).

Although these compounds have already been reported, this is the first report of compounds identified of *N. variegata*. It is also important to note that phenolic compounds that were identified have potential pharmacological properties known, justifying the biological activities that this plant had shown.

Conclusion

In summary, the results obtained complement previous studies, suggesting that *N. variegata* possesses satisfactory antibacterial and photoprotective activities. Phenolic compounds identified in this study are reported for the first time for this species and can justify their biological properties, which can be an important information for the development of analytical methods for the quality control of *N. variegata* using isoquercitrin as chemical marker.

Conflict of Interest

The authors have not declared any conflict of interest.

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